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Ki67 Reactivity in Nasal and Periocular Squamous Cell Carcinomas in Cats Treated with Electron Beam Radiation Therapy

Katja Melzer, Franco Guscetti, Carla Rohrer Bley, Andrea Sumova, Malgorzata Roos, and Barbara Kaser-Hotz

Squamous cell carcinomas of sparsely haired skin are relatively common tumors in cats, and these tumors likely exhibit a rapid growth rate. Thus, we evaluated response and duration of response in relation to the Ki67 proliferative reactivity in such tumors. Seventeen cats with confirmed squamous cell carcinomas and treated with an accelerated, hypofractionated electron beam radiation protocol were included in the study. For all of them histologic grading, Ki67 reactivity, response, and disease-free interval (DFI) were evaluated. Response to therapy was excellent (94% complete response rate) with a median DFI of 414 days. Only moderate acute and few long-term adverse effects were seen. Cats with tumors with a low Ki67 reactivity had markedly shorter DFIs than cats with tumors with high Ki67 reactivity. We concluded that an accelerated, hypofractionated electron beam radiation therapy protocol is well suited for feline squamous cell carcinomas. The protocol appears especially efficacious in tumors with a high Ki67 reactivity.

Key words: Accelerated; Hypofractionated.

Cutaneous squamous cell carcinomas are relatively common tumors in cats and believed to be induced by ultraviolet light. Typical localizations are ears, eyes, and nose, especially in cats with poorly pigmented skin.¹ Many different treatment modalities have been described, such as surgery, radiation therapy, photodynamic therapy, and intralesional chemotherapy^{2–6}. All treatment modalities have been shown to be successful. In contrast to surgery, radiation therapy was found to be a cosmetically appealing and effective option.^{4,6–8}

Squamous cell carcinomas are thought to be rapidly dividing tumors. A potential tumor doubling time of approximately 5 days was determined by using flow cytometry and bromodeoxyuridine (BrdU) staining.⁹ Therefore, an accelerated, hypofractionated electron beam radiation protocol, similar to a previously established proton beam radiation protocol¹⁰ was used and evaluated.

Although BrdU labeling is the most reliable test to evaluate proliferation in tumors, it requires in vivo injection of BrdU before tissue sampling and cannot be performed retrospectively on archival material. A commonly utilized proliferation index is Ki67 immunohistochemistry, which identifies the percentage of Ki67 antigen-expressing tumor cells. The Ki67 antigen is expressed in proliferating cells during all phases of the cell cycle (G1, G2, M, and S phase), but is not found in differentiated, dormant cells in G0. Ki67 methodology has been established in feline formaline-fixed tissue in

apocrine sweat gland tumors, meningiomas, and melanocytic tumors.^{11,12}

The aim of this retrospective study was to evaluate the efficacy of an accelerated, hypofractionated electron beam radiation treatment for feline nasal and periocular squamous cell carcinomas. In addition, the impact of proliferative activity of tumor cells on disease-free interval (DFI) was measured by Ki67 immunohistochemistry.¹³ We hypothesized that tumors with high proliferative activity would respond more effectively and for a longer period of time to accelerated radiation therapy than would tumors with low proliferative activity.

Materials and Methods

Cats included in this retrospective study had electron beam radiation therapy without previous local or medical therapy. Tumors were histologically confirmed cutaneous nasal or periocular squamous cell carcinomas. Records contained information about age, signalment, breed, weight, sex, color of the face, and tumor location for all animals. Data about treatment response and DFI also were available. The 17 cats included in this study were treated between June 2000 and July 2004 in the section of Diagnostic Imaging and Radiation Oncology at the Vetsuisse-Faculty, University of Zurich, Zurich, Switzerland.

All cats were treated by using a linear accelerator^a with a single field, either applied from laterally or dorsocranially depending on the location of the tumor. The energy of the electron beam was chosen depending upon the depth of the tumor and ranged from 5 to 16 MeV. Wet gauze of 2- to 4-mm thickness was used to build up the radiation dose on the skin surface. Treatment planning was done manually. One centimeter was added to the target volume of palpable tumor to decrease risk of underestimating tumor size. A total dose of 48 Gy was given in 10 fractions of 4.8 Gy on 5 consecutive days. To overcome rapid regrowth, 2 fractions at least 6 hours apart were given per day. Beeswax eye shielding was used if indicated to protect the cornea.

To decrease the risk for anemia due to frequent propofol administration, 2 alternating anesthetic protocols were used. Either a midazolam^b and propofol^c or a ketamine^d-valium^e combination was given.

Before therapy, the tumors were staged according to Magne et al² (see Table 1). Tumor size and invasiveness were judged by palpation and histopathology as well as by using visual criteria. Tumor volume was calculated by the formula $\pi/6 \times \text{length} \times \text{width}$

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Table 1. World Health Organization classification of feline tumors of epidermal origin, as modified by Magne et al.²

Tumor Stage	Description
T1a	<1.5-cm diameter, noninvasive
T1b	<1.5-cm diameter, invasive
T2a	>1.5-cm diameter, noninvasive
T2b	>1.5-cm diameter, invasive

× depth.¹⁴ Mandibular lymph nodes were aspirated and cytologically evaluated for regional metastasis, and 2 lateral thoracic radiographs were taken to search for distant metastasis. CBC and serum biochemistry were performed to evaluate of general health status.

Two consecutive histologic tumor sections 30 μ m apart stained with hematoxylin and eosin were evaluated and graded by 1 pathologist (FG). A grading system described by Carpenter et al¹⁵ for squamous cell carcinoma of the tongue in dogs was used with slight modifications. Three grades (I, II, and III) were distinguished, where higher grades indicate more malignant morphology. Four characteristics (lack of differentiation, lack of keratinization, mitotic rate, and nuclear polymorphism) were scored based on the following scale: 0 = not observed, 1 = slight, 2 = moderate, and 3 = extensive. All mitotic figures in ten 400× magnification fields were counted, and the mitotic score was noted as 0 = no mitosis, 1 = <20, 2 = 20–40, and 3 = >40 mitotic figures. Tissue invasion was scored as 0 = none, 1 = predominantly noninfiltrative or well-delineated infiltrating borders, and 2 = infiltrating borders with cellular dissociation in small groups of cells or single cells. Scirrhous reaction was scored as 0 = none or slight and 1 = pronounced. For each tumor, the scores for each characteristic were added and a sum <8 was assigned grade I, 8–10 grade II, and >10 grade III.

A proliferation score was determined by means of immunohistochemistry for Ki-67 antigen (MIB-1). Serial 4- μ m sections were cut and mounted on slides and dried overnight at 37°C. Antigen retrieval was performed by microwaving slides for 10 minutes in citrate buffer at pH 6.0. Immunohistochemical staining was performed automatically in a DAKO Autostainer using the ChemMate system^f by following the manufacturer's instructions. Briefly, 1 section of each sample was incubated with an antibody specific for Ki-67 (clone MIB-1, M7240^g) for 1 hour at room temperature and at a dilution of 1:100. The secondary biotinylated antibodies were applied for 10 minutes, the peroxidase blocking solution for 5 minutes, and the streptavidin peroxidase and the 3-amino-9-ethyl carbazole (AEC)/H₂O₂ substrate solution were applied for 10 minutes each. The slides were rinsed with wash buffer between steps. Finally, the sections were counterstained with hemalum. In most samples, normal epidermis with hair follicles was present adjacent to the tumor tissue and served as a positive and negative control. Positive control staining was found in some basal and in hair bulb keratinocytes, whereas no staining was seen in arrector pili muscles. In addition, a negative control using phosphate-buffered saline instead of the primary antibody was performed on another section of each sample (Fig 1).

Six hundred to 800 cells, except in 1 case (300 cells), were counted in digital images from several randomly chosen regions of each tumor. The percentage of positive cells was determined by dividing the number of positive cells by the total number of cells counted. Score 0 was defined as 0–4% positive cells, score 1 as 5–19%, score 2 as 20–59%, and score 3 as more than 60% positive cells.

Response was evaluated at the time of recheck 3 weeks postradiation. Response was considered as complete when no

tumor was visible and the tissue re-epithelized, as partial when >50% regression was seen, and as no response when tumor had regressed <50% or was growing. DFI was defined as the time from end of therapy until local, regional, or distant failure. Follow-up information was gathered by follow-up examinations, phone conversations, and photographs from owners and referring veterinarians.

Radiation reactions were graded according to the criteria of the Veterinary Radiation Therapy Oncology Group.¹⁶ Reactions occurring within the first 3 weeks after radiation were considered acute. Those observed from 3 months after radiation therapy up to the time the cat was lost to follow-up or died were considered late effects. Acute grade 1 toxicities were defined as erythema and alopecia; grade 2 as erythema, alopecia, and moist patchy desquamation; and grade 3 toxicity as more confluent moist desquamation and edema. Late toxicity was defined as grade 1 if slight alopecia, and grade 2 if slight alopecia and mild fibrosis of the skin were visible. In grade 3 toxicities, severe fibrosis and necrosis of tissue were obvious.

Statistical Analysis

The following parameters were evaluated with help of the StatView statistical analysis software^h: age, weight, sex, anatomic location, tumor volume, laboratory abnormalities, histologic grade, stage of the tumor, Ki67 reactivity, and acute and late toxicities; and DFI was evaluated with simple descriptive statistics¹⁷. A Kaplan-Meier description¹⁷ was used for DFI. For statistical analysis, DFI was chosen over survival, because DFI gives a more precise estimate for tumor control. Some owners keep cats with squamous cell carcinoma alive for a very long time, despite massive tumor growth, whereas others decide soon for euthanasia. The differences in DFI among cats with different scores of Ki67 were investigated by the log rank test.¹⁷ DFI compared to the Ki67-positive reacting cells, the stage of the tumor, and the tumor volume was evaluated by Cox regression.¹⁷ Pearson correlation¹⁷ was computed between Ki67 and stage, grade, age, weight, sex, location, and tumor volume. When the assumption of the bivariate normal distribution was violated, the Spearman rank correlation¹⁷ was computed. It computed between tumor volume and stage, between stage and anemia, and between Ki67 and grade, stage, and tumor volume. Mann-Whitney analysis¹⁷ was used to analyze sex versus age, location versus stage, and Ki67 versus location. Cats were censored from evaluation of DFI for continued life without recurrence or death before recurrence of tumor. DFI was evaluated with a Kaplan-Meier survival plot. Significance was defined as $P < .05$.

Results

Sixteen of the treated cats were Domestic Shorthairs and 1 was a Domestic Longhair. Mean and median ages of the 17 cats were 11.1 and 11.2 years, respectively (range, 8–14 years). Nine were spayed females and 8 were castrated males. Mean and median weight was 4.3 kg. Neither breed nor sex predilection was found.

Anemia was a commonly found laboratory abnormality, with only 3 cats having PCV results within reference limits. The mean PCV was 30.1%, with a range from 21 to 42%. Because almost all cats were anemic, no correlation could be found to DFI. Other abnormalities encountered included slightly increased aspartate aminotransferase and alanine aminotransferase activities (2 cats), neutrophilia (1 cat), increased serum levothyroxine

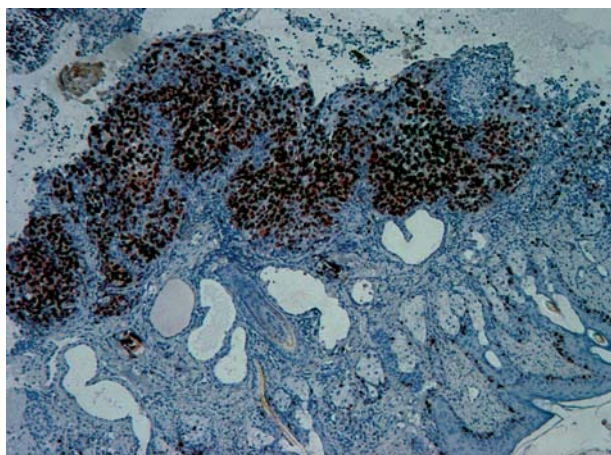


Fig 1. Score 3 Ki67 immunohistochemistry of a nasal squamous cell carcinoma.

concentration (1 cat), and decreased serum albumin concentration (1 cat).

Twelve tumors were located on the nose and 5 periocularly. Median tumor volume was 0.29 cm³, with a range of 0.01–25.4 cm³. Tumors were diagnosed as stage T2b (6 cats), T1b (5 cats), and T1a and T2a (3 cats each). Regional lymph nodes of all cats were free of metastasis. No cat showed evidence of distant metastasis on the thoracic radiographs. Histologically, most tumors were grade 3 (11 cats), 7 cats had grade 2 tumors, and only 1 cat was diagnosed as having a grade 1 tumor.

For Ki67 immunoreactivity an average of 660 cells was evaluated (all but 1 cat [300 cells] had more than 500 cells evaluated). All tumors were positive on Ki67 immunohistochemistry. The range of labeled cells was between 8.3 and 81.6%. The cat with no response to radiation therapy had the lowest count of only 8.3% positively staining cells (Table 2).

All but 1 cat showed complete tumor response. Six cats had local recurrence with very rapid and aggressive growth after recurrence. Three (18%) of 17 cats in this study showed regional or distant metastasis, whereas 2 also had local recurrence at the same time. The other cat was diagnosed 2 months after radiation with regional lymph node metastasis and was treated with excision of the node. No tumor recurrence or further metastasis was detected in the remaining follow-up time of 1,390 days. The remaining 10 cats did not show any recurrence or metastasis and were still alive at the conclusion of the study.

Most acute radiation reactions (10 cats) were grade 1–2 toxicities with erythema, alopecia, and some patchy, moist desquamation. Six cats had grade 2–3 toxicities with more confluent moist desquamation and some edema. In 1 cat, grade 1 toxicity was seen with only mild erythema. Late toxicity was mild to moderate with 13 grade 1 and 4 grade 2 toxicities. Grade 1 toxicity included alopecia, whereas grade 2 was defined as alopecia and fibrosis. In 4 cats, owners reported frequent sneezing episodes in the long-term follow-up >3 months after therapy (Fig 2). No bone necrosis could be seen in any of the cats during the follow-up time. No correlation was found between tumor size or radiation field and severity of toxicity.

Median DFI was 414 days (range, 21–1,450 days). One cat with a Ki67 score = 1 had a DFI of only 21 days. Median DFI for cats with Ki67 score = 2 was 487 days (9 cats), and with score = 3 (7 cats) it was 1,129 days. One- and 2-year DFIs were 66 and 40%, respectively. Higher stage of tumor showed a clear tendency to correlate with shorter DFI ($P = .056$). Although only a small number of cats were included in the study, a significant inverse correlation was found between Ki67 reactivity and DFI ($P = .035$). No significant difference was found between Ki67 reactivity and location of the tumor, tumor volume, stage, or

Table 2. Overview of all treated cats included in the study.

Cat Age (years)	Sex	Tumor Localization	Tumor PCV (%)	Tumor Volume (cm ³)	Stage	Grade	% Ki67-Positive Cells	Ki67 Score	Acute Toxicity (grade)	Late Toxicity (grade)	DFI (days)	Survival (days)	Censored
11	fs	Nose	36	0.690	2	3	60.6	3	3	1	>1,450	SA	No
14	mc	Eye	24	25.400	5	3	47.8	2	3	1	120	120	No
8	fs	Nose	42	0.094	2	2	36.7	2	2	2	>300	SA	No
11	mc	Nose	24	1.942	4	2	58.7	2	2	1	>977	SA	No
8	fs	Eye	26	0.220	2	2	60.6	3	3	1	>987	SA	Yes
12	mc	Nose	34	0.010	1	2	50.2	2	2	1	>987	SA	Yes
12	fs	nose	27	0.722	4	2	45.3	2	2	1	>952	SA	Yes
14	mc	Eye	28	3.140	4	3	8.3	1	2	2	21	LTF	No
12	fs	eye	29	0.285	3	3	73.0	3	2	1	414	414	No
8.5	mc	Nose	35	0.019	2	2	63.8	3	3	0	>623	SA	Yes
8.7	fs	Nose	24	0.026	1	1	74.9	3	2	1	>567	SA	Yes
8	fs	Nose	42	0.031	1	2	42.7	2	2	1	98	124	No
11.5	fs	Eye	29	0.523	4	3	54.2	2	3	1	88	104	No
9.5	mc	Nose	34	0.157	3	3	81.6	3	1	1	>242	SA	No
14	mc	Nose	21	1.569	3	1	46.3	2	2	2	171	171	No
	fs	Nose	43	2.510	4	2	52.7	2	2	1	>300	SA	Yes
15	mc	Nose	35	0.293	2	3	71.0	3	3	2	>494	SA	Yes

DFI, disease-free interval; fs, female, spayed; mc, male, castrated; SA, still alive; LTF, lost to follow-up after 40 days.

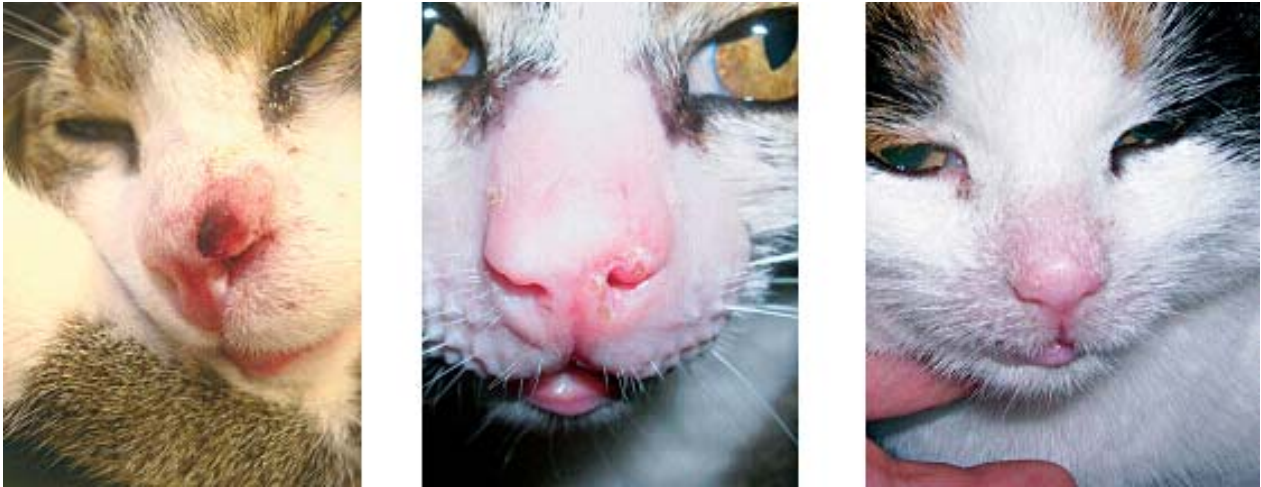


Fig 2. Cat before radiation therapy, after 3 weeks, and after 3 months.

grade. Also in the multivariate analysis with stage, grade, and anatomic location included, Ki67 showed significance only with DFI. A Kaplan-Meier survival plot applying Ki67 score is shown in Figure 3.

Discussion

Rapid tumor proliferation influences response to radiation treatment. Proliferation of surviving tumor cells between radiation fractions may lead to a decreased tumor control. A more rapidly administered radiation protocol will counteract this proliferation. An accelerated protocol implies that the radiation is given over a shorter time than in conventional treatment. Hypofractionation means that larger doses per fraction are given to achieve a shorter overall treatment time, but the risk of delayed toxicity is increased.¹⁰

In a previous study,⁸ an accelerated, hypofractionated proton radiation therapy regimen was shown to be efficacious and well tolerated for the treatment of feline squamous cell carcinomas. DFI of cats in this study was 440 days with a 1-year DFI of 64%. Because of restricted availability of the cyclotron, therapy had to be given within 1 week. Although proton radiation treatment is a modality that is highly conformed to the tumor target, it is unfortunately expensive and not readily available. Therefore, a similar radiation treatment protocol was originally established that used electron beam radiation.

An accelerated, hypofractionated radiation protocol was used in 17 cats with nasal or periocular squamous cell carcinoma. We analyzed the DFI in this radiation therapy protocol and whether the proliferative activity plays a role in the success of such a treatment approach.

This radiation protocol lead to an excellent response rate (94% complete response), which is higher than that observed in other studies, in which between 35 and 60% of patients showed complete response.^{4,7,8} The DFI with a median of 414 days was comparable to that in the studies of Carlisle and Gould with a DFI of 13 months (390 days), Fidel et al⁸ with a DFI of 440 days

(14.6 months), or Théon et al⁴ with a DFI of 16.5 months (502 days). In our study, no control population was treated with a conventional radiation regimen, which makes statements about efficacy difficult. In comparison with results from available literature, including a variety of different radiation protocols, ours appears to be an efficient treatment protocol. The accelerated regimen may have been advantageous for predominantly rapidly dividing, high-grade tumors. The short interval between treatments likely counteracted the rapid regrowth of the squamous cell carcinomas, which has been described as a major factor in treatment failure in fast-growing neoplasms.⁴

Most tumors in this study had a relatively high percentage of Ki67-positive cells on immunohistochemistry, supporting the suspected fast growth pattern of squamous cell carcinomas. No statistically significant difference was found in the amount of positive staining independent of the histologic grade or stage. In squamous cell carcinomas in humans, the labeling index of Ki67 has been reported to correlate with prognosis.¹⁸⁻²¹ A higher labeling index of Ki67 indicates a poorer prognosis with a higher frequency of metastasis. The risk for metastasis could not be evaluated in our study because of the low number of patients. However, DFI was significantly shorter in cats with low Ki67 score. This finding indicates that in cats Ki67 may not necessarily be a negative prognostic factor for metastasis, but it is a positive prognostic factor for treatment response. Nevertheless, the number of cats diagnosed with local or distant metastasis in our study was higher than in other studies.^{2,4-6,8} Whether aggressive treatment could have influenced the metastatic rate (eg. by up-regulating growth factors) is unknown but possible.²²

In our study, a higher proportion of cats had high-grade tumors as compared to other studies. For example, the cats in the study of Théon et al⁴ consisted of equal numbers of grade I, II, and III tumors. This difference was unlikely due to slight difference in the grading system used, but might truly reflect the higher proportion of malignant tumors in our study. Grade was

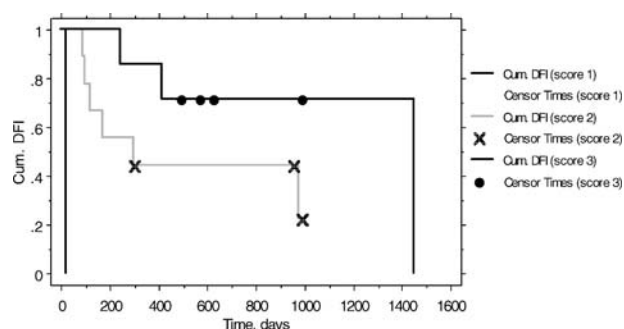


Fig 3. Kaplan-Meier plot for disease-free interval with low (score 1, black), medium (score 2, gray), and high (score 3, dotted) Ki67 activity.

not correlated with stage, localization of tumor, or Ki67 reactivity in the cat population of the present study. Therefore, our findings support the conclusion of the study of Théon et al⁴ study that grade is not a prognostic factor for DFI or response in nasal and periocular squamous cell carcinomas of cats.

Median DFI of cats treated with this radiation regimen was 414 days, with 1- and 2-year DFIs of 66 and 40%, respectively. These numbers are comparable to, or only slightly below, those of previous studies.^{4,7,8} In 3 cats, the question arises if tumor recurrence was true recurrence or new growth of tumor because they occurred long after initial radiation therapy (DFI = 414, 977, and 1,450 days, respectively) and showed a slow growth pattern.

Generally, treatment was very well tolerated, with moderate acute radiation reactions in most cats. The reactions did not markedly differ from those observed in previous studies, although treatment was more aggressive.^{4,7} Most acute adverse effects were resolved by the 3rd week after radiation. Five cats were followed up for >3 years, and no serious late effects such as bone necrosis were detected.

Cats with high proliferative activity had a significantly longer DFI than cats with low proliferative activity. Direct comparison of the different studies is very difficult because many different treatment protocols were used. Our study shows that this accelerated, hypofractionated radiation regimen appears particularly suitable to eradicate fast-growing tumors, because tumors with high Ki67 scores achieved longer tumor control than tumors with low scores. For cats, a short treatment time (ie, time at the hospital) may be advantageous over conventional treatment, because many cats become fractious toward the end of treatment.^{4,7} Additional studies are necessary to evaluate the influence of Ki67 on DFI with the above-mentioned therapy protocol as compared to conventional protocols. Previous studies with a conventionally fractionated protocol⁴ or even prolonged treatment time⁷ were well tolerated and achieved good tumor control, which suggests that duration of treatment may have little influence on control of feline squamous cell carcinomas.

In conclusion, we showed that an accelerated radiation treatment protocol is effective in treating

feline squamous cell carcinomas. Examination of our data indicates that this finding is especially true for tumors with high proliferative activity. Assessment of a proliferation score before therapy could be helpful in the decision of a suitable treatment protocol.

Footnotes

^a Dynaray LA20, ABB/VARIAN, Switzerland

^b Dormicum, Midazolamum, Roche Pharma AG, Reinach, Switzerland

^c Propofol 1% Fresenius, Propofolum, Fresenius Kabi AG, Stans, Switzerland

^d Narketan 10, Ketaminum, Vétoquinol AG, Bern, Switzerland

^e Valium, Diazepamum, Roche Pharma AG, Reinach, Switzerland

^f ChemMate System

^g Clone MIB-1, M7240, DAKO, Zug, Switzerland

^h StatView, Version 5.0.1, SAS Institute Inc, Cary, NC

Acknowledgment

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